# A GLUCOSE ACCEPTOR IN PLANTS WITH THE PROPERTIES OF AN α-SATURATED POLYPRENYL-MONOPHOSPHATE

Rafael Pont Lezica*, Christopher T. Brett**, Pedro Romero Martínez** and Marcelo A. Dankert**

*Departamento de Biología, Fundación Bariloche, San Carlos de Bariloche, Río Negro, and **Instituto de Investigaciones Bioquímicas "Fundación Campomar" and Facultad de Ciencias Exactas y Naturales, Obligado 2490, Buenos Aires (28), Argentina

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SUMMARY: Organic solvent extracts from plants were fractionated by DEAE cellulose column chromatography. Fractions were obtained which acted as glucose acceptors using UDP-Glc as glucose donor and enzymes from both plant and animal origin. The acceptors and their glucosylated derivatives were compared with dolichyl-monophosphate and ficaprenyl-monophosphate by chromatographic and degradative techniques. These tests showed that the acceptors had the properties of α-saturated polyprenyl-monophosphates.

The role of lipid carriers in extracellular polysaccharide synthesis is now well established for microbial systems (1). In mammalian systems, sugar-containing lipids are involved in glycoprotein biosynthesis and their role has been intensively studied recently (2, 3). In each case it has been established that the lipid moiety of the carrier lipid is a polyisoprenoid chain. The chain length ranged from 6 to 21 isoprene residues according to the source employed. Variation also occurred in the number of cis and trans double bonds and the saturation or non-saturation of the alfa residue.

Recent work in higher plants has shown the presence of lipid phosphate sugars. Membrane preparations from Phaseolus aureus incorporated mannose from GDP-($^{14}$C)-Man into lipids, but the evidence for the identity of the lipid moiety was not conclusive (4-6). The transfer of mannose from GDP-($^{14}$C)-Man and glucose from UDP-($^{14}$C)-Glc to lipid phosphate acceptors has also been described in cotton fibers (7). Polyprenyl-phosphates were proposed as the lipid acceptors but their structure was not clearly elucidated. Several polyprenols, including ficaprenol, betulaprenol, and dolichol, have been isolated from plants (1), and any of them (as their phosphorylated derivatives) could have been the endogenous lipid

Abbreviations: DMP: dolichyl-monophosphate; FMP: ficaprenyl-monophosphate.

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acceptors described in those works.

This paper reports the characterization of glucose acceptor lipids from wheat germ, pea, and soybean as compared with ficaprenyl-P and dolichyl-P. The properties of these acceptors are similar to DMP as judged by several criteria.

## MATERIALS AND METHODS

### Isolation of acceptor lipids

a) Soybean and wheat germ acceptors. Two hundred grams of soybean meal (Glicine max) or wheat germ (Triticum vulgaris) were extracted with 300 ml acetone for 3 hr and filtered. The retained material was dried in air, extracted overnight with chloroform-methanol (2:1), and filtered. The filtrate was washed by the method of Folch et al. (8) and then poured into a DEAE-cellulose column (1.2 x 42 cm) in the acetate form (9), together with 50,000 cpm DMP-($^{14}$C)-Glc (prepared from rat liver DMP) as marker. The column was washed with chloroform-methanol (2:1) and eluted with a gradient of 0-0.2 ammonium formate in the same solvent. Fractions of 4.5 ml were collected. Aliquots were counted to detect the DMP-Glc marker; other aliquots were washed by the Folch method and assayed with the rat liver enzyme for the glucose acceptor activity. Omission of the DMP-($^{14}$C)-Glc marker did not change the position or the amount of acceptor activity eluted.

b) Pea acceptor. Dwarf Pisum sativum L. seedlings (var. Cuarentona) were grown in the dark for 5 to 7 days. The plumular hooks were homogenized in an Omni-mixer with 0.1 M Tris-HCl buffer, pH 7.4 and 20 mM β-mercaptoethanol. The homogenate was strained through cheesecloth and centrifuged at 1000 g for 15 min. The resulting supernatant solution was centrifuged at 20,000 g for 1 hr. The pellet obtained was resuspended in the same buffer. This suspension is referred to as "pea enzyme". The pea enzyme was extracted with one volume of butanol three times, and the butanolic phases were combined and washed with water. The butanol extract was passed through a DEAE-cellulose column (1 x 15 cm), and after washing with 99% methanol the active lipid was eluted batchwise using solutions of 0.1, 0.2, and 0.4 M ammonium acetate in the same solvent. The methanol was evaporated under $N_2$, the residue was disolved in butanol and salts were washed out with water.

### Assays for acceptor lipids

a) Rat liver enzyme assay. Rat liver microsomes were prepared as described (10). The sample to be assayed was dried under vacuum with 0.5 μmole MgEDTA and 0.5 μmole $MgCl_2$. To this was added the reaction mixture, which contained 5 μmoles of Tris-maleate, pH 7.8, 5 μmoles of mercaptoethanol, 0.12% Triton X-100, 4 x $10^5$ cpm of UDP-($^{14}$C)-Glc and 5 μl microsomal enzyme in a total volume of 0.05 ml. The system was incubated at 30°C for 30 min, and then 0.4 ml methanol and 0.6 ml chloroform were added. The protein precipitate was removed by centrifugation; the supernatant was washed by the method of Folch (8) to remove UDP-Glc, and counted.

b) Pea enzyme assay. Standard incubations were carried out at 20°C for 30 min in a total volume of 0.05 ml of the following: 50 μmoles Tris-HCl buffer pH 7.4, 10 μmoles β-mercaptoethanol, 0.5 μmole $MgCl_2$, 1 nmole UDP-($^{14}$C)-Glc and pea enzyme (100 to 300 μg of protein).

The reaction mixture was extracted with butanol as described (11). This enzyme preparation always produces both neutral and polar $^{14}$C-glucolipids. They were separated by DEAE-paper chromatography (Whatman DE-20) using butanol as solvent, and the areas of the paper corresponding to the origin (polar lipids) and the solvent front (neutral lipids) were counted.

Chemicals. UDP-($^{14}$C)-Glc (309 mC/mmol) and UDP-($^{3}$H)-Glc (2.42 C/mmol) were synthesized as described previously (11). Ficaprenol was isolated from Ficus elastica leaves according to Stone et al. (12) and phosphorylated by the method of Popjak et al. (13). The purity of the ficaprenol was checked by thin-layer chromatography in systems C and D and by infrared and NMR spectra. Analysis by mass spectrography showed that it was practically 50% undecaprenol and 50% dodecaprenol.

Dolichyl-monophosphate isolated from liver and partially purified, was a gift from Dr. N.H. Behrens.

Chromatography and electrophoresis. Paper chromatography was carried out in Whatman N° 1 paper with: solvent A, 3 M $NH_3$ in 80% ethanol (v/v); solvent B, n-butanol-pyridine-water (6:4:3). For solvent A the ascending technique was followed.

Thin-layer chromatography was carried out on silica gel G plates in solvent: C) isopropyl ether-light petroleum ether (1:4 v/v) (12); D) 1% (v/v) methanol in benzene (12); and E) chloroform-methanol-water (60:25:4).

Paper electrophoresis was performed with Whatman N° 1 paper (11) at 1000 V (20 V/cm) for 3 hr in pyridine acetate 1.2 mM pH 6.5.

Reducing substances were located with the silver nitrate reagent (14).

## RESULTS

Isolation and acid stability of acceptor lipids. Fractionation of the chloroform-methanol extract from soybean on a DEAE column produced a peak of acceptor activity which eluted at an ammonium formate concentration of 0.115 M. The wheat germ extract was fractionated and assayed in the same manner, and gave a pattern of acceptor activity resembling that found in the soybean extract. A similar profile was obtained when DMP was chromatographed in the same way. When the butanol extract from pea was purified by DEAE-cellulose column chromatography, the acceptor activity was found in the 0.1-0.2 M ammonium acetate fraction. FMP was also found to elute in the same concentration range.

Soybean and pea acceptor lipids were treated with acid under various conditions and then assayed for acceptor activity. The results shown in Table I indicate that the plant acceptor lipids and DMP were almost completely stable under the conditions used, while FMP was unstable. After treatment with 0.1 N KOH at 37°C for 1 hr, 92% of the acceptor activity of the soybean lipid still remained. Under the same conditions 84% and 75% of the acceptor activities of DMP and FMP, respectively, were retained.

Properties of the glucosylated acceptor. Mild acid treatment rapidly degraded the glucosylated acceptors. Both DMP-Glc and the glucolipid from soybean were 85% degraded by 0.1 N HCl, 24°C, 10 min. Eighty-seven percent of the pea glucolipid was degraded by 0.01 N HCl, 100°C, 10 min, to give free glucose, as checked by chromatography in solvent B.

TABLE I. Acid treatment of plant acceptors and polyprenol-phosphates.

| Acceptor lipid | Enzyme used for assay | Acid treatment pH | Radioactivity incorporated into acceptors | | % acceptor decomposed |
|---|---|---|---|---|---|
| | | | Untreated cpm | Treated cpm | |
| Soybean | Liver | 1 | 11800 | 11100 | 6 |
| Pea | Pea | 2 | 3211 | 2544 | 21 |
| DMP | Liver | 1 | 11900 | 9300 | 22 |
| DMP | Pea | 2 | 7904 | 6800 | 14 |
| FMP | Liver | 1 | 5600 | 1900 | 67 |
| FMP | Pea | 2 | 5064 | 790 | 85 |

The samples were treated with 0.1 or 0.01 N HCl, 10 min at 100°C. The lipids were extracted by Folch partition or with butanol and then assayed for their acceptor activity with liver or pea assay.

Plant glucolipids and characterized polyprenyl-phosphate sugars were treated with 50% phenol for 3 hr at 68-70°C (11). Under these conditions, FMP-Glc prepared with the pea enzyme, degraded and the label became water soluble (Table II). Electrophoresis of the water phase showed that glucose phosphate was the product. In contrast, soybean and pea glucolipids and DMP-Glc from pig liver were not degraded under these conditions and remained phenol soluble. Similar results were observed by catalytic reduction (15). As shown in Table III, neither DMP-Glc nor glucosylated soybean lipid were degraded by this procedure, but more than 80% of the $C^{14}$ from FMP-($^{14}$C)-Glc became water soluble.

Soybean lipid-P-($^{3}$H)-Glc was co-chromatographed with FMP-($^{14}$C)-Glc and with DMP-($^{14}$C)-Glc on thin-layer plates in solvent E. After development, 0.5 cm wide strips of gel were scraped off the plate and counted. The soybean glucolipid ran exactly the same as DMP-Glc but slightly ahead of FMP-Glc. When DMP-($^{3}$H)-Glc and FMP-($^{14}$C)-Glc were co-chromatographed, DMP-($^{3}$H)-Glc also ran slightly ahead of FMP-($^{14}$C)-Glc (Fig. 1).

The pea glucolipid (prepared using the pea enzyme) was chromatographed in a DEAE-cellulose column and eluted with a gradient of 0-0.4 M ammonium acetate in 99% methanol. The polar glucolipid eluted at 0.125 M ammonium acetate. When the polar lipid was chromatographed in solvent A, only one peak was obtained at the solvent front ($R_f$ 0.95).

TABLE II. The action of phenol on glucosylated lipids.

| Glucolipids | Enzyme used for glucosylation | Phenol phase cpm | Water phase cpm | Degradation % |
|---|---|---|---|---|
| Soybean lipid-P-($^{14}$C)-Glc | Liver | 1889 | 201 | 10 |
| Pea lipid-P-($^{14}$C)-Glc | Pea | 3028 | 292 | 9 |
| DMP-($^{14}$C)-Glc | Liver | 1046 | 126 | 10 |
| FMP-($^{14}$C)-Glc | Pea | 514 | 2342 | 82 |

Samples were treated with 50% phenol at 68-70°C for 3 hr and the water and phenol phases were separated as described (11). Aliquots from both phases were counted. Recovery of radioactivity was at least 80%.

## DISCUSSION

Both the liver enzyme and the pea enzyme used in this work were able to use either DMP or FMP as glucose acceptors. In the case of the liver enzyme, this is in agreement with the results obtained with UDP-Glc (10) or with GDP-Man as donor (6, 1, 17). This lack of specificity enabled us to use these enzymes as general assay systems to detect polyprenyl-phosphates.

Acidic lipids that act as glucose acceptors in these enzyme systems were isolated from plant extracts (wheat germ, soybean, and peas) fractionated by DEAE-cellulose column chromatography. The fact that the acceptors are acidic suggests that the lipids were polyprenyl-phosphates, since other acceptors, such as sterols, would be uncharged. The stability of the soybean acceptor lipid to mild alkali rules out the possibility that it might be a fatty acid ester. Further evidence for the polyprenyl-phosphate nature of the acceptor lipids was provided by the elution pattern from the DEAE-cellulose columns, which in each case coincided with polyprenyl-monophosphate makers (DMP or FMP). In addition, the glucosylated acceptors obtained with either pea or liver enzymes were labile to mild acid, as glucosylated polyprenyl phosphates are. The $R_f$ (0.95) of the pea glucolipid in solvent A indicates that it was resistant to alkaline conditions, as would be expected for a monophosphate compound. A lipid pyrophosphate glucose would give the cyclic phosphate ester derivative ($R_f$ 0.66). This result is in agreement with the pattern obtained by DEAE-cellulose column chromatography, since the elution of a peak at a concentration of 0.125 M ammonium acetate fits in well with the results obtained with polyprenyl-monophosphate glucose in other systems (18). Under the conditions used, a pyrophosphate derivative would have

TABLE III. Catalytic reduction.

| Sample* | Butanol phase cpm | Water phase cpm | Degradation % |
|---|---|---|---|
| Soybean lipid-P-($^{14}$C)-Glc | 4590 | 112 | 3 |
| DMP-($^{14}$C)-Glc | 5700 | 35 | 1 |
| FMP-($^{14}$C)-Glc | 900 | 3440 | 79 |

Reduction was carried out with $H_2$ gas and Platinum catalyst (15) in water saturated butanol for 4 hr. Radioactivity was measured in a scintillator after butanol-water partition. Recoveries were at least 87%.

*The soybean lipid-P-($^{14}$C)-Glc and FMP-($^{14}$C)-Glc samples contained 8% and 14% of DMP-($^{14}$C)-Glc, respectively, as calculated from the amounts of endogenous DMP present in the liver enzyme preparation used to synthesize these compounds.

been eluted in the 0.2-0.3 M range (11). It seems highly probable, therefore, that the acceptors obtained from plants were polyprenyl-monophosphates.

The plant acceptors were treated with acid to determine whether or not the phosphate was present in an allylic linkage. They were found to be stable to mild acid conditions, as was DMP, while in contrast FMP lost its acceptor activity. More evidence for the saturation of the α-isoprene residue of the plant acceptors was given by the stability of the glucosylated acceptors to phenol hydrolysis and to catalytic hydrogenation. In each case ficaprenyl-P-Glc, like other allylic polyprenyl-phosphate-sugars (18), was found to be unstable under the same conditions. In contrast, it was found that DMP-Glc was stable to both treatments.

This α-saturation suggests a similarity between the plant acceptors and dolichyl-phosphate. Thin-layer chromatography of the glucolipids provided further evidence that the plant acceptors are of the dolichyl-phosphate type rather than the ficaprenyl-phosphate type. Soybean glucolipid and DMP-Glc had the same mobility, while FMP-Glc consistently ran more slowly. This difference possibly reflects a difference in chain length, which would indicate that the soybean lipid is the same size as DMP. However, the exact length of the polyprenol chain has not been established.

The fact that the glucose acceptor in plants is similar to dolichyl-phosphate and is clearly not ficaprenyl-phosphate, is of considerable interest, since the free polyprenol present in plants is generally of the allylic type (1). Only in one rather specialised plant tissue has dolichol been reported (19). It may be, therefore, that the ficaprenol found in plants has a role unrelated to the forma-

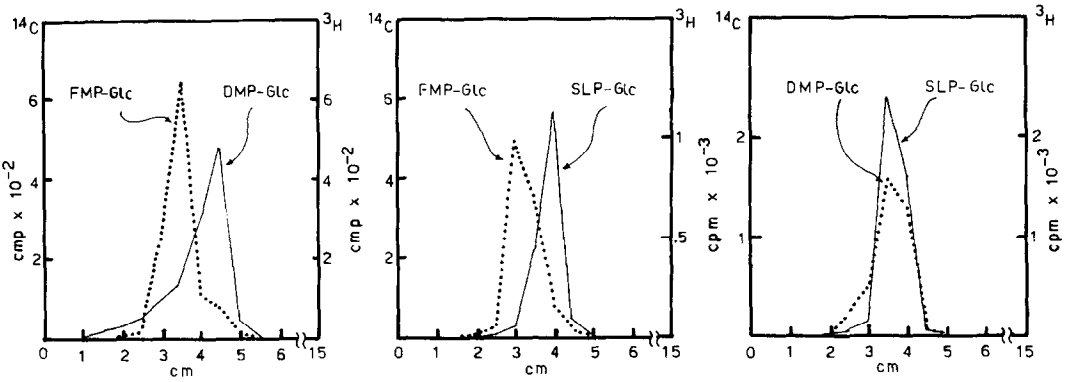


Fig. 1. Thin-layer chromatography on silica gel G plates and solvent E. SLP-Glc: soybean lipid phosphate glucose.

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tion of polyprenyl-phosphates that act as sugar acceptors. These results also suggest that the presence of sugar acceptors with the properties of α-saturated polyprenyl-monophosphates may be a general feature of eucaryotic cells, since they have now been found in yeast, higher plants, and a variety of animal tissues. In contrast, the sugar acceptors characteristic of procaryotic cells are α-unsaturated polyprenyl-phosphates (1, 2, 3).

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